Rheumatoid arthritis patients have a pro-atherogenic cytokine microenvironment in the aortic adventitia.

Running title: Cytokines in Aortic Adventitia of RA patients

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**Background:** People with rheumatoid arthritis (RA) are at increased risk of developing cardiovascular disease (CVD) via as yet, poorly defined mechanisms. Inflammatory pathways, in particular within the vascular adventitia, are implicated in primary CVD pathogenesis but could be amplified in RA at the local tissue level. We therefore examined aortic adventitial (AA) from coronary artery disease (CAD) patients with or without RA to determine the cytokine profile contained therein.

**Methods and Results:** AA and internal mammary artery (IMA) biopsies from 19 RA patients and 20 non-RA patients undergoing coronary artery bypass graft surgery (CABG) were examined by immunohistochemistry. IL-18, IL-33 and Tumor Necrosis Factor (TNF) were expressed in AA biopsies in both groups, and their expression was significantly higher in RA patients. In RA patients, IL-33 expression in endothelial cells correlated positively with the number of swollen joints, suggesting a link between the systemic disease state and the local vascular tissue microlesion.

**Conclusions:** The presence of pro-inflammatory cytokines IL-18, IL-33 and TNF may play a role in the inflammatory process within the adventitia that contributes to plaque formation and destabilization. In theory, the amplified expression of these cytokines may contribute to the known increased occurrence and severity of CAD in RA.

**Key Words:** rheumatoid arthritis; atherosclerosis; biopsy; inflammation; cytokines
Rheumatoid arthritis (RA) is associated with increased risk of cardiovascular disease (CVD), which cannot be completely explained by traditional risk factors (1). Cardiovascular events in RA are characterized by greater severity in terms of recurrence and mortality. It is likely that both increased plaque formation and vulnerability plays a role in the increased CV morbidity in RA.

The proposed mechanisms of initiation and perpetuation of RA as well as atherosclerosis are multifactorial, and have yet to be fully elucidated. Both diseases share immunological/inflammatory components in their postulated pathology. Inflammation plays an important role in all stages of the atherosclerotic process (from endothelial cell activation to plaque formation, destabilization and thrombosis) (2). Levels of systemic inflammatory biomarkers, including C-reactive protein (CRP), TNF and IL-18, predict CVD, and/or disease progression in the general population. Consequently, there has been increasing interest in the role of cytokines in vascular disease, and the systemic levels of many cytokines, including IL-33, have been investigated in this context. It should be appreciated, however, that the role of circulating IL-33 is complicated and thought to be situation dependent. In mouse models of RA, administration of exogenous IL-33 enhances disease activity whilst in models of atherosclerosis it is protective (3-5). Moreover, soluble ST2 (sST2), the IL-33 soluble receptor, is emerging as a novel biomarker of adverse vascular outcome. Administration of excess sST2 has been shown to exacerbate atherosclerosis in mice (3), suggesting that circulating IL-33 expression in the local lesion may indeed be a protective factor.

We previously reported that among patients with CVD, those with inflammatory rheumatic diseases have a higher occurrence of inflammatory infiltrates in their aortic adventitia (AA) (6). Furthermore, compared to the aorta, the occurrence of the adventitial infiltrates was significantly lower in the internal mammary artery (IMA), which is a vessel highly resistant to atherosclerosis. The adventitial inflammation, involving vasa vasorum, may play a substantial effect in plaque formation, progression, destabilization and contribute to the increased cardiovascular (CV) risk in RA. In keeping with this notion, current research has indicated a reduction of CV morbidity or mortality in patients effectively treated with anti-rheumatic therapies, which might be at least partially mediated through inhibition of vascular inflammation. In support of this concept, a recent study has demonstrated a significant
attenuation of vascular inflammation in RA patients treated with anti-TNF therapy (7). Thus, with the introduction of new immunological therapies, it is essential to examine which immune factors are involved in the vascular inflammatory process as this may have implications for prevention and treatment of CVD.

We hypothesised that an amplified cytokine milieu exists within vascular lesions in patients with RA compared to controls, thus providing a potential tissue-based explanation for the accelerated vascular risk that ensues in RA. Therefore, the aim of this study was to examine inflammatory cytokines, with plausible bio-effects in the vasculature, in the aortic adventitia of CVD patients with and without RA, and evaluate their relationship to informative clinical parameters. We elected to focus on IL-18, IL-33 and TNF, since they have been implicated in both atherosclerosis and RA pathology.

METHODS

Patients

Of patients undergoing coronary artery bypass graft (CABG) surgery in the Feiring Biopsy Heart Study (6), we selected 19 with RA fulfilling the American College of Rheumatology 1987 criteria and 20 random controls. All the patients gave written informed consent. Regional Ethics committee for Medical Research Norway approved the study. Demographics and clinical characteristics are shown in Table 1.

Human biopsy and immunohistochemistry

We examined specimens from AA (which include the aortic part of the epicardial layer) and IMA taken during CABG. The AA (approximately 5x10 mm), covered by the periaortic part of the epicardium, was removed from the ventral aspect of the ascending aorta, in connection with the establishment of the proximal aorto-coronary anastomoses (6). For patient’s safety, these anastomoses are made (and, therefore, the biopsies are taken) at areas with less pronounced atherosclerosis. The tissue from IMA (5-10 mm long) was taken from the distal part of IMA, while the proximal part was
used as a graft (8). The biopsies were fixed in formalin and embedded in paraffin, and cut in 3µm thick sections. Some findings from AA and IMA used in this study have already been reported (6,8).

**Immunohistochemistry**

Slides were prepared as previously described (6). In brief, depending on antibodies (Supplement Table 1), slides were pre-blocked with 0.5% hydrogen peroxide, followed by antigen retrieval (citrate heat retrieval or trypsin). After blocking with 5% normal horse serum ± biotin, tissue sections were incubated with primary antibodies (CD68, IL-18, IL-33 and TNF) overnight, followed by 0.5% hydrogen peroxide if required. Secondary HRP-conjugate or biotin-conjugated secondary antibodies were incubated for 1h, followed by avidin-biotinylated peroxidase macromolecular complex (ABC Vector Labs). Binding was visualized with ImmPACT DAB peroxidase substrate (Vector Labs). Slides were counterstained with hematoxylin.

**Scoring of stained tissues**

Slides were semi-quantitatively scored in a random order by disease-blinded researchers. CD68 were scored as follows: 0 = no cells, 1 = <20 cells, 2 = 21-50 cells, 3 = 51-99 cells, 4 = 100-199 cells, 5 = >200 cells per section. IL-18 and TNF were scored using the following score: 0 = 0% of section, 1 = <10% of section, 2 = 10-25% of section, 3 = >25% of section. IL-33 was scored both according to the percentage of IL-33 positive vasa vasorum per section, and according the number of IL-33 positive endothelial cells (EC) per vasa vasorum.
Soluble autoantibody and cytokine receptor detection

All serum samples were collected prior to CABG, after a minimum of 4 hours fasting. ELISA, using appropriately diluted serum as per manufacturer’s instructions, was used to assay soluble ST2 (R&D systems) and anti-CCP2 (Axis-shield) titres. The BN™ Systems N Latex RF kit (Siemens Healthcare Diagnostic) was used to determine rheumatoid factor (RF) levels in serum samples.

Statistical analysis

GraphPad Prism and SPSS were used for all statistical analyses. The chi-square test and Fisher’s exact test (for categorical variables), the t-test for independent samples (for continuous normally distributed variables), and the Mann-Whitney U test (for continuous variables without normal distribution) were used to identify differences between RA and non-RA patients. To determine correlations, Spearman’s rho test was used. The level of statistical significance was set at 0.05, and all tests were 2-sided.
RESULTS

Macrophages, IL-18 and TNF expression in AA

The proportion of AA biopsies in which CD68\(^+\) macrophages were detectable (63% RA and 56% non-RA) and the amount of CD68\(^+\) cells per section (Figure 1A, Supplemental Figure 1A) was similar in RA and non-RA patients with CVD. A greater number of biopsies from RA patients (63%) expressed TNF compared to non-RA patients (30%)\((P = 0.04)\) (Supplemental Figure 1A), whereas IL-18 was present in all biopsies suggesting ubiquitous expression in the aortic vascular lesion (Supplemental Figure 1A). Crucially, although there was no difference in the number of macrophages (Figure 1A), the absolute level of IL-18 and TNF expression in AA was higher in RA patients (Figure 1B-C) \((P = 0.03\) and \(P = 0.02\), respectively) compared with non-RA controls. Neither the number of macrophages, nor the level of IL-18 or TNF expression correlated with hypertension or smoking (Supplemental Table 2). Furthermore, their expression did not correlate with several CVD severity markers, including the number of coronary arteries with significant stenosis, New York Heart Association (NYHA) class or the number of previous myocardial infarctions. However, in RA patients, the left ventricular ejection fraction as assessed by ventriculography negatively correlated with IL-18 levels \((r = -0.569; P = 0.03)\) and macrophage number \((r = 0.587; P = 0.05)\). In RA patients, neither macrophage nor cytokine expression correlated with several markers of disease severity, activity (i.e., DAS28, tender joint count, ESR, CRP, RF or anti-CCP) or disease duration.

IL-33 in endothelial cells (EC) within AA

IL-33 was detected in the majority of AA biopsies in both RA (88%) and non-RA (72%) patients, but was restricted to the nucleus of vasa vasorum EC (Supplemental Figure 1B). The proportion of vasa vasorum expressing IL-33 (IL-33\(^+\) VV) was higher in RA than non-RA patients (20.4% ±15.8 versus 8.6% ±9.5; \(P = 0.02\)) (Figure 2A). The proportion of EC nuclei positive for IL-33 within each vasa vasorum (IL-33\(^+\) EC) was also substantially greater in RA than non-RA patients (58.7% ±34.1 versus 32.9% ±28.5; \(P = 0.02\)) (Figure 2B). In IMA, the occurrence of IL-33\(^+\) VV was similar in RA (89%) and non-RA (79%) patients but RA patients had a higher proportion of IL-33\(^+\) EC (30.6% ±26.3 versus 13.1% ±12.5; \(P = 0.04\)). IL-33\(^+\) EC and IL-33\(^+\) VV in AA did not correlate with any traditional...
CV risk factors (Supplemental Table 2). In RA, the proportion of IL-33+ EC in AA significantly correlated with the NYHA functional classification ($r = 0.618; P = 0.018$) but not other CVD severity markers. Furthermore, in RA, the proportion of IL-33+ EC in AA also significantly correlated with tender joint counts ($r = 0.582; P = 0.023$) and swollen joint counts ($r = 0.619; P = 0.014$) (data not shown and Figure 2C).

Interpretation of the functional implications of circulating IL-33 expression generally requires parallel evaluation of its soluble regulatory receptor, sST2. Even though we only observed nuclear IL-33 expression in EC and not extracellular, we evaluated serum sST2 levels and found it to be significantly higher in RA than non-RA patients (158.5pg/ml ±52 versus 12.79pg/ml ±13) ($P = 0.003$) (Figure 2D). The level of sST2 did not correlate with vascular IL-33 expression or other RA clinical and immunological parameters, however, it did correlate with expression of IL-18 ($r = 0.582; P = 0.02$) and CRP ($r = 0.745; P = 0.001$).
Discussion

Our novel study demonstrates a significant alteration in the AA cytokine microenvironment of CAD patients with RA compared to those without RA. Our data suggests that the underlying pathology in the vessel is different not only at a local level (i.e., EC of microvessels within AA) but also systemically within other macrovessels (i.e., IMA). We found that compared with non-RA controls, RA patients have significantly higher expression of the pro-inflammatory cytokines IL-18 and TNF in AA, higher expression of nuclear IL-33 in EC of vasa vasorum in AA, and higher levels of sST2.

Although most studies have focussed on circulating IL-33 expression, it has previously been shown to be expressed in human vascular EC (3)(9), and this may be of great importance. Intracellular/nuclear full length IL-33, which is thought to act as an ‘alarmin’ when released by necrotic cells (10), is a transcription factor (11). As a transcription factor, endothelial IL-33 can induce NFκB signalling, with subsequent EC activation (i.e., up-regulation of VCAM and ICAM) (12). Thus, the vasa vasora of RA patients with increased nuclear IL-33 are likely to be more predisposed to EC activation leading to increased adhesion and infiltration of inflammatory cells that again will increase IL-33 expression. This is further supported by the fact that TNF (increased in AA of our RA cohort) is known to stimulate EC with a consequent up-regulation of ICAM and VCAM via an intracellular IL-33-dependent signal (12). The increased IL-33 expression within microvascular EC of RA patients might therefore represent a stromal alteration, which could contribute to increased CVD risk in RA.

Furthermore, it is known that TNF, similar to other pro-inflammatory cytokines, increases the secretion of sST2 from numerous cell types (13). Thus, although sST2 may counteract circulating IL-33 activity, the high systemic levels of sST2 in RA could reflect enhanced inflammation, which is supported by the observed correlation with CRP. Moreover, the correlation of sST2 with AA IL-18 is also pertinent given that increased IL-18 exacerbates atherosclerosis (14) while depletion reduces atherosclerosis (15). Collectively, our data suggests that within RA patients, the adventitial microenvironment, characterized by higher levels of IL-18 and TNF and a higher expression of nuclear IL-33 within microvascular endothelial cells, together with increased levels of sST2, amplify the pro-atherogenic nature of the vessel, which theoretically enhances atherosclerotic pathology.
Based on the observed increase of IL-33 in EC both within the AA and IMA of RA patients, one might speculate that this phenomenon could lead to a generalized endothelial dysfunction and impairment of the microvascular function, which is likely to contribute to both RA-specific manifestations (such as synovitis and extra-articular manifestations) and to the pathogenesis of ischemia (due to both macro- and microvascular disease), and heart failure. In theory, the observed positive association with NYHA class may reflect, among other factors, an increased ischemia in patients with increased IL-33 expression in EC, causing microvascular cardiac disease (which is known to frequently occur in RA).

It should also be appreciated that although the luminal endothelium has been the main focus of studies and theories to explain aspects of the pathogenesis of atherosclerosis, our studies suggest that the endothelium covering vasa vasorum (which provide the oxygen and nutrition supply to the macrovessel) may also play a crucial role in the development of atherosclerosis.

Of note, the number of macrophage and level of IL-18 expression in the AA was inversely related to left ventricular ejection fraction. The cause of the finding is unclear, and although this could be the result of a Type-2 error, it could reveal an important clinical relationship. For example, the aortic inflammation might reflect the systemic inflammation, which might contribute to heart failure development. Moreover, it is possible that the aortic inflammation mirrors a more generalized vascular inflammation, including coronary arteries (and small intramural cardiac arteries), which leads to myocardial ischemia with a consequent ischemia-related heart failure.

The current study has several possible limitations, which include a relatively small sample-size, and hence the lack of some associations and differences may be caused by Type-2 errors. However, compared to other biopsy studies, this study is relatively large and does provide justification for larger more in-depth studies. This is also a cross-sectional study and therefore no cause/effect relationships can be identified. However, it should be appreciated that this is the first study to examine the AA microenvironment in RA and non-RA patients, and thus provides valuable information about vascular biology. Moreover, an additional advantage of this study is that it does not rely on autopsy samples,
which are susceptible to deterioration postmortem but rather uses difficult to obtain fresh surgical specimens.

In summary, our data suggest that not only systemic but also vascular inflammation may play a role in the pathogenesis of CVD, and of accelerated CVD in RA, with IL-33 and related cytokines as potentially important player. These findings may help to explain why anti-rheumatic therapies, including anti-TNF therapy, decrease CVD risk(7). In theory, these therapies may reduce the CV risk not only due to its effect on systemic inflammation, but also due to its direct effect on the vasculature: both on inflammation in plaques, and on adventitial inflammation, which is suspected to be involved in both atheroma formation and destabilization. These results therefore emphasize the necessity for future studies that examine the influence of the cytokine vascular environment in RA on CV outcome.
Acknowledgements

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Disclosures

None.

Figure 1. IL-18, TNF in AA of patients with late stage CVD with and without RA. Immuno-histological evaluation and quantitation of (A) CD68⁺ macrophage, (B) IL-18, and (C) TNF in AA of patients with CVD with (n = 19) or without RA (n = 20). Results are shown as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 5th and 95th percentiles. * = P < 0.05.

Figure 2. IL-33 and sST2 in patients with late stage CVD with and without RA. Immuno-histological evaluation and quantification of (A) the percentage of vasa vasorum positive for IL-33 (B) the percentage of IL-33⁺ EC in each vasa vasorum, in AA of patients with CVD with (n = 19) or without RA (n = 20). Results are shown as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 5th and 95th percentiles. (C) Correlation between swollen joint counts and the percentage of IL-33⁺ EC in each vasa vasorum in RA patients. A spearman rank correlation coefficient (r) and p value is provided to quantify the associations. (D) Serum levels of soluble ST2 (sST2). Bars show the mean ± SEM. * = P < 0.05, *** = P = 0.002.


14. Whitman SC, Ravisankar P, Daugherty A. Interleukin-18 enhances atherosclerosis in

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>RA (n=19)</th>
<th>Non-RA (n=20)</th>
<th>P value</th>
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<tr>
<td>Age – years</td>
<td>69±9.1</td>
<td>68±9.5</td>
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<td>Males – no. (%)</td>
<td>12 (63)</td>
<td>14 (70)</td>
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<td>Duration of CAD – years</td>
<td>6.3±8.7</td>
<td>7.2±6.6</td>
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<td>History of myocardial infarction – no. (%)</td>
<td>15 (79)</td>
<td>9 (45)</td>
<td>0.05</td>
</tr>
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<td>Acute coronary syndrome – no. (%)</td>
<td>9 (47)</td>
<td>4 (20)</td>
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<tr>
<td>Time from angiography to CABG – days</td>
<td>17±30</td>
<td>31±60</td>
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<tr>
<td>Left ventricle ejection fraction</td>
<td>59±13</td>
<td>64±13</td>
<td>0.16</td>
</tr>
<tr>
<td>Hypertension – no. (%)</td>
<td>13 (68)</td>
<td>9 (45)</td>
<td>0.20</td>
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<tr>
<td>Family history of CAD – no. (%)</td>
<td>11 (58)</td>
<td>20 (100)</td>
<td>0.001</td>
</tr>
<tr>
<td>Hyperlipidemia – no. (%)</td>
<td>16 (84)</td>
<td>20 (100)</td>
<td>0.11</td>
</tr>
<tr>
<td>Duration of RA – years</td>
<td>19±15</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Patient global assessment of IRD (VAS, 0-100 millimeters)</td>
<td>29±24</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Number of swollen joints (0-28)</td>
<td>2.2±2.6</td>
<td>0</td>
<td>0.002</td>
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<tr>
<td>DAS28</td>
<td>3.2±1.0</td>
<td>-</td>
<td>-</td>
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<tr>
<td>RF (IU/ml)</td>
<td>316±568</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CCP (U/ml)</td>
<td>553±667</td>
<td>-</td>
<td>-</td>
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<tr>
<td>C-reactive protein – mg/liter</td>
<td>20±39</td>
<td>3.1±3.1</td>
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<td>Erythrocyte sedimentation rate – mm/hour</td>
<td>34±29</td>
<td>15±9.5</td>
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<td>Body mass index – kg/m²</td>
<td>25±5.0</td>
<td>25±2.4</td>
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<td>Diabetes – no. (%)</td>
<td>2 (11)</td>
<td>1 (5)</td>
<td>0.61</td>
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<td>Previous smoker – no. (%)</td>
<td>10 (52)</td>
<td>12 (60)</td>
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<tr>
<td>Current smoker – no. (%)</td>
<td>2 (11)</td>
<td>1 (5)</td>
<td>0.61</td>
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<tr>
<td>Current use of:</td>
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<td></td>
<td></td>
</tr>
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<td>Oral glucocorticosteroids – no. (%)</td>
<td>8 (42)</td>
<td>0 (0)</td>
<td>0.001</td>
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<tr>
<td>Disease modifying drugs – no. (%)#</td>
<td>16 (84)</td>
<td>0 (0)</td>
<td>&lt;0.0001</td>
</tr>
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<td>Cox2-selective inhibitors – no. (%)</td>
<td>5 (26)</td>
<td>0 (0)</td>
<td>0.02</td>
</tr>
<tr>
<td>Traditional NSAIDs – no. (%)</td>
<td>2 (11)</td>
<td>0 (0)</td>
<td>0.23</td>
</tr>
<tr>
<td>Lipid-lowering drugs – no. (%)</td>
<td>16 (84)</td>
<td>19 (95)</td>
<td>0.34</td>
</tr>
<tr>
<td>Acetylsalicylic acid – no. (%)</td>
<td>17 (90)</td>
<td>17 (85)</td>
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</tr>
<tr>
<td>Beta blockers – no (%)</td>
<td>15 (88)</td>
<td>14 (74)</td>
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</tr>
<tr>
<td>ACE inhibitors – no. (%)</td>
<td>5 (31)</td>
<td>5 (25)</td>
<td>0.47</td>
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We used the Fisher exact unconditional test to assess differences in proportions between the two groups. To examine differences in continuous variables between the two groups, we used the independent samples t-test (age, BMI), the Mann-Whitney U-test (duration of CAD, Time from angiography to CABG, LVEF, CRP, ESR), the Wilcoxon Signed Rank Test (number of swollen joints).
Figure 1. IL-18, TNFα in AA of patients with late stage CVD with and without RA. Immuno-histological evaluation and quantitation of (A) CD68+ macrophage, (B) IL-18, and (C) TNFα in AA of patients with CVD with (n = 19) or without RA (n = 20). Results are shown as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 5th and 95th percentiles. * = P < 0.05.

83x80mm (300 x 300 DPI)
Figure 2. IL-33 and sST2 in patients with late stage CVD with and without RA. Immuno-histological evaluation and quantification of (A) the percentage of vasa vasorum positive for IL-33 (B) the percentage of IL-33+ EC in each vasa vasorum, in AA of patients with CVD with (n = 19) or without RA (n = 20). Results are shown as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 5th and 95th percentiles. (C) Correlation between swollen joint counts and the percentage of IL-33+ EC in each vasa vasorum in RA patients. A spearman rank correlation coefficient (r) and p value is provided to quantify the associations. (D) Serum levels of soluble ST2 (sST2). Bars show the mean ± SEM. * = P < 0.05, ** = P = 0.002.